Biosynthetic Conversion of Thebaine to Codeine[1]

Harriet I. Parker,[2] Gottfried Blaschke,[3] and Henry Rapoport*

Contribution from the Department of Chemistry and Lawrence Radiation Laboratory, University of California, Berkeley, California 94720.
Received June 23, 1971

Abstract: The conversion of thebaine (I) to codeine (II) by *Papaver somniferum* has been studied in detail. In particular, we have used a short-term steady-state method for conducting $^{14}CO_2$ exposures which avoids ambiguity in discerning precursor–product relationships. This method combined with feeding experiments has established that codeinone (VI) and neopinone (VIII) are involved in the biosynthesis of codeine (II). The feeding experiments also demonstrated that codeine methyl ether (III) can be converted into codeine; however, no codeine methyl ether could be found as a natural constituent of the plants. Fed codeinone (VI) and neopinone (VIII), both were converted into codeine by the plants. The natural presence of both of these compounds in small amounts was demonstrated by $^{14}CO_2$ exposures followed by isolation of radioactive material. Codeinone's role was further confirmed by short-term steady-state exposure to $^{14}CO_2$. The specific activities of thebaine, codeinone, and codeine after such an exposure were in the sequence thebaine > codeinone > codeine, as required for the intermediacy of codeinone in the conversion of thebaine to codeine. The experiments reported here lead to the conclusion that thebaine is converted to codeine by initial demethylation to neopinone, followed by rearrangement to codeinone which is reduced to codeine.

The final steps of the biosynthesis of morphine by *Papaver somniferum* have been shown[4,5] to involve the conversion of thebaine (I) to codeine (II), followed by demethylation of codeine to morphine (IV). This sequence was determined using $^{14}CO_2$ exposures[4] and precursor feedings,[5] which also demonstrated that both of these steps are irreversible. We have now investigated the conversion of thebaine to codeine in more detail. At least one intermediate must be involved in this transition, since two processes occur: demethylation and reduction. If demethylation occurs first, then either neopinone (VIII) or codeinone (VI), or both, would be intermediates. However, if the first step is reduction of thebaine, then codeine methyl ether (III) would be involved in the conversion to codeine.

Previously[6] we presented evidence for the role of codeinone (V) as an intermediate in the conversion of thebaine (I) to codeine (II). We have now refined our approach and methods to eliminate any possible sources of ambiguity in biosynthetic studies of this type. In addition, we now present evidence regarding the role of neopinone (VIII) in the biosynthesis of codeine.

In general, the approaches currently used in studying biosynthetic sequences fall into two categories: precursor feedings and exposure to (or growth in) an isotopically labeled environment. Both methods potentially present the possibility of alteration of the system being studied, thus perhaps leading to aberrant results.

Precursor feedings using various techniques (for example, injection,[7] root feeding,[6] wick feeding,[8] or smearing on the leaves[9]) subject the plant to an unnatural situation in that the site of feeding and concentration of substrate are not normal, and the plant may alter its metabolic behavior as a consequence. Therefore, conclusions based solely on such feedings should be regarded with some reservation.

Growth in an isotopically labeled environment circumvents some of these problems. For plants this is conveniently obtained by exposure to $^{14}CO_2$; however, interpretive errors may result if the exposures are not conducted under steady-state conditions. Exposure experiments of this type determine if a specific compound is a natural constituent of the plant, such evidence reflecting on its possible involvement in a biosynthetic scheme. More definitive data result when the specific activity of each of several compounds in the proposed path is analyzed. For these studies to be valid, steady-state conditions must exist.

The requirements for this steady-state condition to obtain are two: (1) all growth conditions other than the presence of $^{14}CO_2$ must be normal; (2) the specific activity of the $^{14}CO_2$ must not decrease during the exposure. To satisfy the first requirement, factors such as pressure, lighting, carbon dioxide concentration, humidity, and nutrient must all be at their natural levels. Most of these conditions are easily controlled, but control of the carbon dioxide concentration has been much more difficult. As a consequence, most of the exposures have started out with a quite high carbon dioxide concentration (0.15–0.2%, whereas air-level concentration is 0.04%) in order to ensure enough carbon dioxide for the length of the exposure, and the concentration has then been allowed to decrease steadily during the course of the exposure, sometimes to a concentration lower than that of air level. Although in some cases the $^{14}CO_2$ is added in pulses to prevent too high concentrations, continuous control of the carbon dioxide concentration has not been achieved. Such control is desirable to eliminate any distortion of normal metabolic paths that might result from high or low carbon dioxide concentrations.[10]

(1) Supported in part by the U. S. Atomic Energy Commission and Grant MH 12797 from the National Institute of Mental Health, U. S. Public Health Service.
(2) National Institutes of Health Predoctoral Fellow.
(3) Fellow of the Deutsche Forschungsgemeinschaft.
(4) H. Rapoport, F. R. Stermitz, and D. R. Baker, *J. Amer. Chem. Soc.*, **82**, 2765 (1960).
(5) F. R. Stermitz and H. Rapoport, *ibid.*, 83, 4045 (1961).
(6) G. Blaschke, H. Parker, and H. Rapoport, *ibid.*, **89**, 1540 (1967).
(7) *E.g.*, A. R. Battersby, R. Binks, and B. J. T. Harper, *J. Chem. Soc.*, 3534 (1962).
(8) E. Leete, *Science*, **147**, 1000 (1965).
(9) G. Kleinschmidt and K. Mothes, *Z. Naturforsch.*, **14b**, 52 (1959).
(10) T. A. Pedersen, M. Kirk, and J. A. Bassham, *Physiol. Plant*, **19**, 219 (1966); I. Zelitch, *J. Biol. Chem.*, **234**, 3077 (1959); **240**, 1869 (1965).

The condition that the specific activity of the $^{14}CO_2$ must not decrease is necessary when specific activities of several compounds of a proposed sequence are to be compared. For example, if A → B → C, until all pools are saturated, the specific activity of A will be higher than that of B, which in term will be higher than that of C, if steady-state conditions apply and the specific activity of the $^{14}CO_2$ has not decreased (Figure 1a). However, if the specific activity of the $^{14}CO_2$ has decreased during the exposure, any number of relationships among the specific activities of the three compounds in question could be possible (Figure 1b).[11] It is to avoid such ambiguity that the second requirement is necessary.

If both requirements are achieved during the total course of the exposure, *i.e.*, (1) normal growth conditions and (2) constant $^{14}CO_2$ specific activity, then the only possible artifact introduced by the probe would result from an isotopic effect or radiation damage. Both of these have been shown to cause no deviations from normal at our operational levels;[12] therefore, such exposures may be considered a completely normal reflection of the plant's chemistry. We have now developed a system in which plants can be exposed to $^{14}CO_2$ under these conditions, and the procedure is described in detail in the Experimental Section.

In studying the biosynthetic conversion of thebaine to codeine, we have used both precursor feedings and exposures to $^{14}CO_2$. Since none of the compounds whose role we were investigating had been detected previously in opium poppies, our first experiment with each compound was to feed the ^{14}C-labeled compound to the plant and examine incorporation of radioactivity into codeine and morphine. When such incorporation was detected, then a more exacting search was conducted for the compound as a natural constituent in the plant, followed by a comparison of specific activities among precursors and products as final evidence.

We initially examined the codeine methyl ether—codeinone question to determine which route was followed in the conversion of thebaine to codeine. Preliminary results have been presented;[6] additional data and details are presented now.

Precursor feeding experiments with codeine methyl ether and codeinone employed nuclear-labeled compounds in order to avoid questions arising from possible transmethylations. Each compound was prepared from randomly labeled [^{14}C]morphine (IV) obtained from 2-day biosynthesis in a $^{14}CO_2$ atmosphere. The morphine was converted to normorphine *via* cyanogen bromide on heroin (V).[13] To prepare codeine methyl ether (III), normorphine was methylated with dimethyl sulfate,[14] converted to the quaternary chloride, and sublimed to eliminate methyl chloride and yield the final product. Nuclear-labeled codeinone (VI) was prepared from normorphine by initial N-methylation to morphine *via* O^3,*N*-dicarboethoxynormorphine and subsequent reduction with lithium aluminum hydride,[15] followed by O^3-methylation to codeine (II) using diazomethane, and finally oxidation with silver carbonate to codeinone.[16]

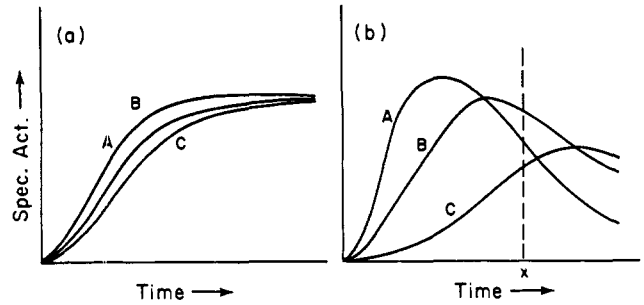
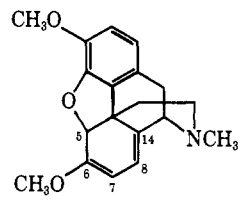
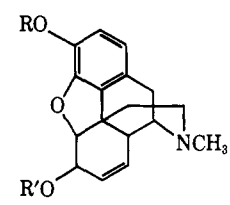


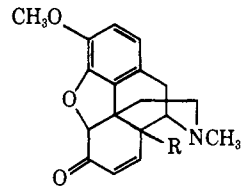
Figure 1. Relative specific activities in a biosynthetic sequence A → B → C when (a) the $^{14}CO_2$ specific activity remains constant; (b) the $^{14}CO_2$ specific activity decreases during the exposure period.



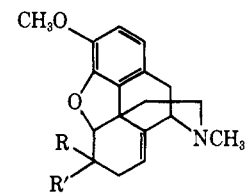
I



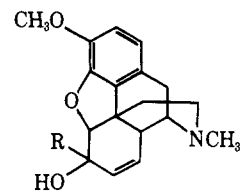
II, R = CH_3; R′ = H
III, R = R′ = CH_3
IV, R = R′ = H
V, R = R′ = CH_3CO



VI, R = H
VII, R = Br



VIII, R = R′ = =O
IX, R = H; R′ = OH
X, R = CH_3; R′ = OH



XI, R = CH_3
XII, R = C_6H_5

Feeding was through the roots, as this method probably produces the least damage to the plants. However, one factor which must be considered in root feedings is alteration of the compound being fed in the nutrient solution during feeding, either chemically or by microorganisms on the roots of the plant. If a compound is altered and then directly taken into the plant, there is no way of detecting this change. However, examination of the nutrient solution and an acid wash of the roots after the feeding established that all the radioactivity recovered is still in the form of the compound fed. In addition, isolation of the fed compound itself from the plant is another indication that incorporation *via* the roots is probably of the compound fed and not of some altered form of that compound.

(11) D. B. Zilversmit, C. Entenman, and M. C. Fishler, *J. Gen. Physiol.*, **26**, 325 (1943).
(12) J. W. Weigl, P. M. Warrington, and M. Calvin, *J. Amer. Chem. Soc.*, **73**, 5058 (1951).
(13) J. von Braun, *Ber.*, **47**, 2312 (1914).
(14) C. Mannich, *Arch. Pharm.*, **254**, 349 (1916).
(15) E. Elison, H. W. Elliott, M. Look, and H. Rapoport, *J. Med. Chem.*, **6**, 237 (1963).
(16) H. Rapoport and H. N. Reist, *J. Amer. Chem. Soc.*, **77**, 490 (1955).

Table I. Feeding Experiments[a] with ^{14}C-Nuclear Labeled Codeine Methyl Ether, Codeinone, and Codeine

Compd fed	Amount fed, dpm	Amount fed, mg/100 g of plant	Incorpn, %	% of incorporated activity in compounds isolated: Thebaine	Codeinone	Codeine methyl ether	Codeine	Morphine
Codeine methyl ether	74,000	12.1	71			52	4.7	3.0
Codeinone	380,000	7.8	81	0	14[b]		14.5	2.8
Codeine	130,400	12.0	61		0[c]		54	13

[a] Using four 64-day-old plants of *P. somniferum*, 3 g each, fed hydroponically *via* nutrient solution. Labeled candidate precursors were added at zero time and growth was continued for 24 hr, adding nutrient as needed to maintain a constant level. The plants were then removed, their roots were washed with 0.1 *M* H_3PO_4, and alkaloids were isolated by the usual procedure,[4] adding inactive thebaine, codeine methyl ether, codeinone, codeine, and morphine as carrier as needed. [b] Analyzed as dihydrocodeinone. [c] Analyzed as 6-methylcodeine.

Table II. Search for Codeine Methyl Ether, Codeinone, and Neopinone in *Papaver somniferum* Plants[a] after $^{14}CO_2$ Exposure

Compd sought	Compd isolated[b]	Carrier added, mg	Spec act. of isolated compd, dpm/mg	Total act. in compd, dpm	% of thebaine act.
Codeine methyl ether	Thebaine	0	2.8×10^7	1.9×10^8	
	Codeine	0	3.5×10^6	1.8×10^8	94[c]
	Codeine methyl ether	33		324	<0.02
Codeinone	Thebaine	62	1.8×10^5	11×10^8	
	Codeine	60	2×10^4	1.2×10^8	10.9
	Codeinone[d]	298	2×10^3	6×10^5	5.4
Neopinone	Thebaine	58	2.1×10^6	12×10^7	
	Codeine	50	1.5×10^6	7.5×10^7	62
	Neopinone[e]	240	3.1×10^4	7.4×10^7	6.1

[a] The plants used were 53–66 days old, weighed 15–25 g, and were exposed to about 50 mCi of $^{14}CO_2$ over a total period of about 4 hr during which approximately 90% of the radioactivity was absorbed. [b] Isolated in the usual way,[4] followed by tlc, gc, and crystallization to constant spec act. (see Experimental Section). [c] No carrier was added in this determination, hence no correction could be made for loss during work-up, therefore this value should not be compared with those in the other two experiments. [d] Characterized by hydrogenation to dihydrocodeinone after separation as its bisulfite addition product. [e] Characterized as 6-methylneopine.

Following the feedings, the plants were subjected to the usual isolation procedure[4] in order to separate the phenolic and nonphenolic alkaloids. Column chromatography was used to separate thebaine, codeine, and codeine methyl ether, and these compounds were further purified by thin layer chromatography, sublimation, and recrystallization to constant activity. In the case of the codeinone feeding, since codeinone decomposed somewhat when subjected to thin layer or column chromatography, it was separated from the other alkaloids through its bisulfite addition product,[17] and was then hydrogenated to dihydrocodeinone[18] for further purification.

The results of the feeding experiments are presented in Table I. Both codeinone and codeine methyl ether are incorporated into codeine and morphine when they are fed to the plant, as was reported previously by us[6] and subsequently confirmed by others.[19-21] These data by themselves suggest several possibilities but provided few conclusions. It is clear that *P. somniferum* can use both codeinone and codeine methyl ether when these compounds are administered to the plants. However, does this indicate dual pathways from thebaine to codeine? Another possibility is that one of these pathways is aberrant, induced by the presence of an unnatural but closely related molecule, and converted to codeine by nonspecific demethylating or reducing enzymes. From these data alone no further conclusions can be drawn.

Further clarification was obtained from $^{14}CO_2$ exposures. Initially,[6] these exposures were conducted under conditions which did not satisfy the requirements for steady state described earlier in this paper, but subsequently the system described above was developed. The experiments conducted under non-steady-state conditions were used solely to detect the presence of either codeine methyl ether or codeinone in the plant. These results are presented in Table II. We could find no codeine methyl ether in the plants using a combination of gas chromatography, with flame ionization detector, and liquid scintillation counting. These methods were sufficiently sensitive to have detected codeine methyl ether in as low a quantity as 0.02% of the thebaine found in the plants. However, we did find codeinone to the extent of 5% of the amount of thebaine present. These experiments, combined with the feeding experiments (Table I), give strong support to the biosynthetic route being thebaine (I) → codeinone (VI) → codeine (II) → morphine (IV).

More definite evidence for the role of codeinone in the biosynthesis of codeine is provided by the steady-state exposure to $^{14}CO_2$. The plants were kept in the exposure chamber for 3 hr before removal and isolation and no carrier was added. After isolation of the nonphenolic alkaloids, codeinone was separated from the other alkaloids by means of its bisulfite adduct and was then treated with phenyllithium to form 6-phenyl-

(17) H. Rapoport, C. H. Lovell, H. N. Reist, and M. E. Warren, *ibid.*, 89, 1942 (1967).
(18) C. Mannich and H. Loewenheim, *Arch. Pharm.*, 258, 295 (1920).
(19) A. R. Battersby, E. Brochmann-Hanssen, and J. A. Martin, *Chem. Commun.*, 483 (1967).
(20) A. R. Battersby, J. A. Martin, and E. Brochmann-Hanssen, *J. Chem. Soc., C*, 1785 (1967).
(21) E. Brochmann-Hanssen, B. Nielsen, and G. Aadahl, *J. Pharm. Sci.*, 56, 1207 (1967).

codeine (XII) for subsequent analysis.[22] The masses of all three compounds were determined by gas chromatography. Aliquots of the sample also were collected and then counted by liquid scintillation to obtain activity determinations. Collecting efficiency was first determined for each compound. In the case of thebaine, some decomposition occurs on gc, but no loss of label occurs as determined by trapping and counting standard samples of labeled [^{14}C]thebaine. The specific activities of the compounds calculated from these data are presented in Table III.

Table III. Specific Activities of Thebaine, Codeinone, and Codeine Isolated from *P. somniferum*[a] after Steady-State Exposure[b] to $^{14}CO_2$[c]

Compd[d]	Spec act., dpm/μmol × 10^{-7}	Wt/100 g of plant, μg
Thebaine	24	85
Thebaine minus O^6–CH_3[e]	18	
Codeinone[f]	16	0.2
Codeine	6	135

[a] Nine 64-day old plants, *ca.* 30 g each. [b] Exposed for 3 hr in the light. [c] The atmosphere in the exposure chamber was maintained at 0.04% carbon dioxide concentration, specific activity ~0.2 mCi/ml, and 29 mCi of $^{14}CO_2$ was absorbed. [d] Isolated in the usual manner,[4] without carrier; final purification by gc, followed by collection and liquid scintillation counting. [e] Determined by conversion to 14-bromocodeinone. [f] Determined as 6-phenylcodeine.

One further determination was needed before the results from the steady-state exposure could be fully interpreted. The O^6–CH_3 group of thebaine (I) does not exist in either codeinone (VI) or codeine (II). A valid comparison of the specific activities of the three compounds isolated after the exposure requires that all three compounds have their carbon atoms in common. In order to determine the activity in the thebaine which was due to the O^6–CH_3 group, a portion of the nonphenolic alkaloid mixture was mixed with unlabeled thebaine and submitted to column chromatography. The isolated thebaine was purified to constant activity and treated with *N*-bromosuccinimide to produce 14-bromocodeinone (VII).[23] Comparison of the specific activity of the 14-bromocodeinone to that of thebaine established that 24% of the activity of thebaine had been in the O^6–CH_3 group which was lost in the conversion to 14-bromocodeinone. This corrected value for the specific activity of the thebaine, eliminating the activity contributed by the O^6–CH_3 group, was then used for comparison with codeinone.

Table III shows that the relative specific activities of thebaine, codeinone, and codeine follow the required pattern for a biosynthetic sequence of thebaine → codeinone → codeine; *i.e.*, the specific activity of thebaine is higher than that of codeinone, which is higher than that of codeine. In addition, the specific activity of codeinone is very close to that of thebaine, which is what would be expected for the immediate precursor–product relationship where the product is of small pool size.

An interesting observation concerns the specific activity of the O^6–CH_3 of thebaine which, within experimental error, is equal to the specific activity of the $^{14}CO_2$ in the exposure chamber. This fact indicates that the O^6–methyl group is put on a very immediate precursor of thebaine (or that the pool sizes of the immediate precursors are very small), and that the turnover of thebaine is quite rapid, since this position is reported not to undergo transmethylation.[24] The order of methylation and the lability of these methyl groups has received little attention in the study of thebaine biosynthesis. Such studies may provide significant information on the biosynthesis and biochemical role of thebaine.

One further question exists regarding the role of codeinone in the conversion of thebaine to codeine, and that is the possibility of an equilibrium between thebaine and codeinone or between codeinone and codeine since it is already known that the overall conversion of thebaine to codeine is not reversible.[6] Upon feeding labeled codeinone to the plant, no radioactivity was found in the thebaine, thus demonstrating that the conversion of thebaine to codeinone is irreversible. Failure to observe any transmethylation at O^6 in thebaine[24] also supports irreversibility at this step. In addition, nuclear-labeled codeine was fed and no radioactivity could be detected in the codeinone (Table I), confirming previous[19,20] observations of this irreversibility.

The conversion of codeine methyl ether to codeine now presents an interesting case. Possibly, codeine methyl ether is a true intermediate in the conversion of thebaine to codeine, but occurs in an extremely small pool. This is highly improbable as no codeine methyl ether could be detected down to a limit of 0.02% of the thebaine present, whereas codeinone had a pool size equivalent to 5% of that of thebaine. Another possibility is that codeine methyl ether is converted back to thebaine, which is then converted to codeine, but no activity could be detected in thebaine after labeled codeine methyl ether had been fed.[21] It would appear, then, that the conversion of codeine methyl ether to codeine is an aberrant path, resulting from demethylation by a nonspecific demethylating enzyme.

With codeinone established as an intermediate in the conversion of thebaine to codeine, the mechanism of the conversion of thebaine to codeinone becomes of interest. Thebaine is the enol ether of both codeinone (VI) and neopinone (VIII). Codeinone is thermodynamically more stable than neopinone; however, this fact does not discount the possibility that initial demethylation of thebaine may first produce neopinone, which then rearranges to the more stable codeinone. If this were the case, it might also help to explain the partial loss of tritium at C-7 which has been reported[24a] in the conversion of thebaine to codeine.

Neopinone (VIII) was prepared[23] by conversion of thebaine (I) to 14-bromocodeinone (VII) followed by hydrogenation to yield neopinone hydrobromide. Care must be exercised in the liberation of free neopi-

(22) Dihydrocodeinone was not used, as it had been in the feeding experiment, since hydrogenation of very small amounts of codeinone led to the formation of numerous side products. 6-Phenylcodeine, on the other hand, was formed in quantitative yield and also had a retention time on gc (which was used for the analysis) quite distinct from the retention times of the other nonphenolic alkaloids.

(23) H. Conroy, *J. Amer. Chem. Soc.*, 77, 5960 (1955).

(24) (a) D. H. R. Barton, G. W. Kirby, W. Steglich, G. M. Thomas, A. R. Battersby, T. A. Dobson, and H. Ramuz, *J. Chem. Soc.*, 2423 (1965); (b) R. O. Martin, M. E. Warren, and H. Rapoport, *Biochemistry*, 6, 2355 (1967).

Table IV. [^{14}C]Neopinone and [^{14}C]Codeinone Feedings[a] to *P. somniferum*[b]

Compd fed	Amount fed: dpm	Amount fed: mg/100 g of plant	% Incorpn, total	% of incorpd activity in compounds isolated: Codeinone	Codeine	Morphine
Neopinone[c]	88,000	80	49	19[d]	8.0	8.7
Codeinone[e]	240,000	106	57	2.3[f]	16	4.7
Codeinone[e]	101,160	14	72	2.5[f]	17	3.4

[a] Feedings were conducted for 4 hr, followed by 24 hr of additional growth. [b] Six 58-day-old plants, each weighing 5 g, were used for each experiment. [c] Carrier neopinone, codeine, and morphine added in isolation. [d] Recovered as a mixture of neopinone and codeinone, converted to codeinone by alumina chromatography, and characterized as 6-methylcodeine. [e] Carrier codeinone, codeine, and morphine added in isolation. [f] Characterized as 6-methylcodeine.

none as a small amount of codeinone is formed quite readily. In fact, this contamination by codeinone presented a major problem in our biosynthetic studies. We found that even at pH 9 a significant amount of codeinone was formed from neopinone after 1 hr. The stability of neopinone in the nutrient solution was also studied, and after 4 hr an increase of ~5% in the codeinone content was detected.

Confirmation that the neopinone contained some codeinone was obtained from a number of sources. Reaction with borohydride resulted in neopine (IX) containing up to 20% codeine. Reaction with methyllithium gave 6-methylneopine (X) and also 6-methylcodeine[25] (XI). Further evidence was found on formamide-impregnated cellulose tlc,[26] and a quantitative measure was supplied in the nmr by the absorption of the C-5 H, δ 4.69 for codeinone and 5.00 for neopinone.

Several approaches were used in an attempt to obtain codeinone-free neopinone. Crystallization as a chloroform adduct[23] led only to a slight decrease in the codeinone content. Alumina[27] and silica gel chromatography converted most of the neopinone to codeinone. Chromatography on paper and on formamide-impregnated cellulose[26] effected a separation, but some neopinone was isomerized to codeinone during the elution process. Removal of codeinone *via* its bisulfite adduct[17] was also attempted, but the recovered neopinone still contained a small contaminant of codeinone. Since 5% of the neopinone was converted to codeinone in the nutrient solution (pH 5.5) during a feeding experiment (and perhaps also in the plant's fluids), further attempts at purification were discontinued.

With the instability of neopinone in mind, a feeding experiment was designed as follows. Three feedings were conducted in parallel, on plants of the same age and size. Six plants were fed [^{14}C]neopinone which have been prepared from biosynthetic [^{14}C]thebaine. Six other plants were fed approximately the same mass of [^{14}C]codeinone. Finally, other plants were fed a mass of [^{14}C]codeinone corresponding to 15% of the neopinone fed. Thus, in the last feeding the codeinone fed was equal to or slightly greater than the amount of codeinone in the neopinone fed. All three feedings were conducted for 4 hr, after which the plants were allowed to grow for another 24 hr in fresh nutrient solution. Prior to isolation, carrier codeinone, codeine, and morphine were added to the codeinone-fed plants and carrier neopinone, codeine, and morphine were added to the neopinone-fed plants. In the two codeinone feedings, codeinone was separated from codeine by the bisulfite procedure and was then converted to 6-methylcodeine (XI). In the neopinone feeding, the neopinone was converted to codeinone by chromatography on alumina,[27] and the codeinone was separated from codeine as above. Purification of all compounds was accomplished primarily by tlc.

The results from these feedings are presented in Table IV. It can be seen that the incorporation of activity from neopinone into codeine and morphine was much greater than could be attributed to the codeinone impurity. Since neopinone is slowly converted to codeinone in aqueous solution over a wide pH range, it could be argued that neopinone, once incorporated into the plant, continued to undergo such conversion, and the codeinone thus formed was converted to codeine. Thus conversion of neopinone to codeinone might not be part of a natural biosynthetic route, but merely the result of neopinone's instability.

To answer this question, we examined *P. somniferum* for the presence of neopinone. For this purpose a derivative of neopinone was sought which would uniquely come from neopinone, and two were considered: neopine (IX) and 6-methylneopine (X). Although neopine has not been isolated from fresh plants, the possibility of its existing there is present. In fact, such a possibility becomes large if neopinone were present in the plant, as it could be converted to neopine by simple reduction in the same manner as codeinone is converted to codeine. To avoid any ambiguity, we searched for neopinone in the plant by converting it to 6-methylneopine. To establish that no interference occurred from other alkaloids present, [^{14}C]thebaine, [^{14}C]codeine, and [^{14}C]codeinone were all treated with methyllithium and then 6-methylneopine was added. No activity appeared in the 6-methylneopine fraction isolated.

The search for neopinone was conducted with plants grown for 2 days in a $^{14}CO_2$ environment. Carrier neopinone, codeine, and thebaine were added to the plant material and the entire nonphenolic alkaloid fraction was treated with methyllithium. Thebaine, codeine, 6-methylneopine, and 6-methylcodeine were separated by means of alumina tlc, the thebaine, codeine, and 6-methylneopine being purified to constant activity. The results are presented in Table II.

This experiment has demonstrated that neopinone is a natural constituent of *P. somniferum*. The earlier questions regarding the feeding experiment can now be answered. It is known that neopinone rearranges to codeinone in aqueous solution, and probably this can happen inside the plant as well as *in vitro*. Thus a

(25) S. P. Findlay and L. F. Small, *J. Amer. Chem. Soc.*, 72, 3249 (1950).
(26) K. Teichert, M. Mutschler, and H. Rochelmeyer, *Deut. Apoth.-Ztg.*, **100**, 477 (1960).
(27) F. Krausz, and T. Rüll, *Bull. Soc. Chim. Fr.*, 2148 (1960).

feeding experiment showing that neopinone is converted to codeine in the plant may have no particular significance, since the codeine may have derived from codeinone. Now that neopinone has been found naturally occurring, it is reasonable to assume that neopinone is converted to codeinone and thence to codeine as a normal process.

From Table II, it would appear that neopinone and codeinone have approximately the same pool size. However, since neopinone is known to be quite unstable, and readily rearranges to codeinone, even during the isolation procedure, a significant amount of the codeinone reported to have been found in the plants may actually have been formed in this manner. In fact, the point could be raised that perhaps codeinone does not exist in the plant and that all of the codeinone which has been isolated came from rearrangement of neopinone during isolation. However, mechanistically, it is very difficult to conceive of a route by which neopinone could be reduced to codeine without prior rearrangement to codeinone. Reduction first to neopine, followed by rearrangement of neopine to codeine, is a very unlikely possibility, particularly since the rearrangement of neopinone to codeinone is known to occur readily, whereas a similar rearrangement of neopine to codeine has never been observed.

The data presented indicate that the most probable route for the conversion of thebaine to codeine is one involving initial demethylation to neopinone, rearrangement to codeinone, and finally reduction to codeine. The observed[24a] loss of tritium at C-7 during the conversion of dual-labeled thebaine to codeine also may be explained by this route if a nonspecific addition and/or removal of hydrogen at C-7 occurs during conversion to neopinone and codeinone. Another possibility is that the route thebaine → neopinone → codeinone → codeine is a minor route, the major one being thebaine → codeinone → codeine. At present, we are unable to clarify this point any further; however, the data establish the involvement of both neopinone and codeinone in the biosynthesis of codeine and morphine.

Experimental Section[28]

Codeinone (VI) was prepared by the Ag_2CO_3 oxidation of codeine.[16]

6-Methylcodeine (XI) was prepared from codeinone as directed,[25] mp 114–115° (lit.[25] mp 114.5–116.5°).

6-Phenylcodeine (XII). Codeinone (1 g) was dissolved in 40 ml of toluene and excess phenyllithium solution was added. After standing at 0° for 1 hr, the reaction mixture was treated with water, acidified with 0.1 *M* phosphoric acid, and extracted three times with toluene. The aqueous phase was then made strongly alkaline and extracted with chloroform. Evaporation of the dried chloroform solution and tlc gave 1.1 g, 90% yield, of 6-phenylcodeine as a glass: uv λ_{max} 285 nm (ϵ 2080); nmr same as codeine[29] with the absence of the C-6 H (4.2) and presence of a multiplet for the C-6 phenyl (7.3); mass spectrum *m/e* 375 (M^+).

Anal. Calcd for $C_{24}H_{25}O_3N$: C, 76.8; H, 6.7; N, 3.7. Found: 76.6; H, 6.6; N, 3.7.

Neopinone (VIII) was prepared from thebaine as directed.[23]

6-Methylneopine (X). Neopinone (1.2 g) dissolved in 20 ml of toluene was allowed to react with excess methyllithium at room temperature for 0.5 hr. Water was added, the toluene layer was removed, and the aqueous phase was extracted twice with toluene. Evaporation of the dried toluene solutions left a residue which was purified by tlc on alumina followed by sublimation at 65° (40 μ): mp 106–108°; mass spectrum *m/e* 313 (M^+); uv λ_{max} 283 nm (ϵ 2100); nmr same as neopine[29] with the absence of the C-6 H (4.23), 1.34 (s, C-6 CH_3), 4.37 (s, C-5 H).

Anal. Calcd for $C_{19}H_{23}O_3N$: C, 72.8; H, 7.4; N, 4.4. Found: C, 72.6; H, 7.3; N, 4.3.

Preparation of Labeled Compounds for Precursor Feeding Experiments. Randomly labeled morphine, obtained by exposure of *P. somniferum* to $^{14}CO_2$, was diluted with radioinactive morphine and converted to [^{14}C]normorphine as directed.[15]

[*nuclear*-^{14}C]**Codeine methyl ether** was prepared by exhaustive methylation of the normorphine with dimethyl sulfate,[14] followed by ion exchange (AGX1, 200–400 mesh, Cl^- form), evaporation of the eluate, and sublimation. The sublimate was chromatographed on alumina, eluting with chloroform and chloroform—1% ethanol, to give [*nuclear*-^{14}C]codeine methyl ether, mp 140–141° (lit.[14] mp 140–141°).

[*nuclear*-^{14}C]**Codeine** was prepared by first converting[15] the [^{14}C]normorphine to [*nuclear*-^{14}C]morphine and then treating the latter with diazomethane.

[*nuclear*-^{14}C]**Codeinone** was prepared by Ag_2CO_3 oxidation[16] of [*nuclear*-^{14}C]codeine.

[^{14}C]**Neopinone** was prepared as directed[23] from [^{14}C]thebaine obtained by exposure of *P. somniferum* to $^{14}CO_2$.

Precursor Feeding Procedure. The plants to be fed were placed in hydroponic solution[30] for 4 days prior to the feeding experiment. On the day of the feeding, they were transferred individually to darkened 25-ml erlenmeyer flasks. The sample to be fed was dissolved in 0.5 ml of 0.1 *M* H_3PO_4 and then in 12 ml of nutrient solution, and 2 ml was fed to each plant. Aliquots were also taken for counting and small additional amounts of nutrient were added to each flask over a period of 4 hr. At this time, all of the nutrient was removed from the flasks and the roots of each plant were washed with fresh nutrient. The flasks were then filled with fresh nutrient; the plants were allowed to grow until the next day when they were removed, the roots were washed with dilute acid, and the plants then were frozen in liquid nitrogen and subjected to the isolation procedure.

Procedure for $^{14}CO_2$ Exposures. The $^{14}CO_2$, generated from $Ba^{14}CO_3$, is trapped in a brass cylinder of small volume (25 ml) immersed in liquid nitrogen using the vacuum line technique. Since the $^{14}CO_2$ generated is of very high specific activity, it is diluted by separate addition of a known amount of $^{12}CO_2$. Alternatively, if it is necessary to keep the $^{14}CO_2$ at a high specific activity, pressure can be increased in the cylinder by addition of nitrogen. The rate of flow out of the cylinder and into the exposure chamber is regulated by a needle valve so as to maintain normal air-level concentration of carbon dioxide in the exposure chamber. Carbon dioxide must be added constantly as it is consumed by the plants. If a small number of plants is used, as is frequently the case, this rate is usually less than 1 ml/min.

An experiment is conducted by first freeing the air in the exposure chamber of carbon dioxide by circulation through ascarite for several hours. The $^{14}CO_2$ and $^{12}CO_2$ from cylinders are added to the chamber to air-level concentrations of carbon dioxide. Some $^{12}CO_2$ is added at the beginning of the experiment to allow for the initially respired carbon dioxide which would have a much lower specific activity than that in the exposure chamber and hence would dilute the specific activity of the gas in the exposure chamber. To ensure that the overall specific activity of the $^{14}CO_2$ in the chamber will not decrease during the exposure, the $^{14}CO_2$ added has a higher specific activity than that originally present. To accomplish this original dilution, some $^{12}CO_2$ is mixed with the original $^{14}CO_2$ used to fill the chamber. All carbon dioxide added subsequently is from the $^{14}CO_2$ cylinder.

Once the exposure chamber has reached air-level concentration of carbon dioxide (now ^{14}C labeled), the plants are admitted *via* a dual-door system. The entire experiment is conducted in the light, $^{14}CO_2$ being admitted to the chamber from the cylinder as it is needed to maintain air-level concentrations of carbon dioxide in the chamber throughout the exposure time. Regulation of the $^{14}CO_2$ in the exposure chamber is monitored by circulating the air

(28) Melting points are uncorrected. Infrared spectra were taken in chloroform solution and are reported in cm^{-1}. Ultraviolet spectra were taken in methanol, and nuclear magnetic resonance spectra were obtained in deuteriochloroform and are reported as δ values downfield from internal tetramethylsilane (δ 0). Radioactivity was determined by scintillation counting. Elemental analyses and mass spectra were provided by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley.

(29) S. Okuda, S. Yamaguchi, Y. Kawazoe, and K. Tsuda, *Chem. Pharm. Bull.*, **12**, 104 (1964); T. J. Batterham, K. H. Bell, and U. Weiss, *Aust. J. Chem.*, **18**, 1799 (1965).

(30) D. R. Hoagland and T. C. Brayer, *Plant Physiol.*, **11**, 471 (1936).

in the chamber through a sensitive Liston-Becker infrared CO_2 analyzer and through a vibrating reed electrometer. Humidity is maintained at greenhouse level by occasional circulation of the chamber atmosphere through Drierite, and normal lighting is supplied by Gro-Lux fluorescent lights.

Isolation Procedures. The alkaloids were isolated by the usual procedure,[4] involving freezing the plants in liquid nitrogen, extraction into butanol—benzene, transfer into acid, then pH adjustment and extraction to obtain two fractions: the phenolic and nonphenolic alkaloids. In the experiments in which carrier alkaloids were added during the work-up, these were dissolved in butanol–benzene and added to the frozen plant material during the initial extraction.

Since morphine was the only phenolic alkaloid of interest, it was separated from the other phenolic alkaloids by column chromatography or tlc, system a (see Chromatography section), depending on the quantity of material. Further purification was accomplished by sublimation followed by recrystallization from methanol to constant specific activity.

In the fraction containing the nonphenolic alkaloids, codeinone was separated from the other compounds by means of its bisulfite adduct.[17] It was then converted to a more stable compound for further purification and analysis as dihydrocodeinone,[18] 6-methylcodeine,[25] or 6-phenylcodeine, as indicated in the tables. Purification of dihydrocodeinone was accomplished by sublimation and recrystallization from methanol. 6-Methylcodeine was sublimed, subjected to tlc systems a, b, and c, and recrystallized. 6-Phenylcodeine, which was the compound used to determine the specific activity of codeinone in the steady-state exposure (Table III), was analyzed by means of gas chromatography.

Codeine and thebaine were separated from each other by means of column chromatography, then sublimed and recrystallized to constant activity. In the case of the steady-state exposure (Table III), these compounds were separated and analyzed by gc.

Neopinone was immediately converted to 6-methylneopine by treating the entire nonphenolic alkaloid fraction with methyllithium. A preliminary test was conducted to establish that no contamination of 6-methylneopine occurred from other substances in the alkaloid mix. The 6-methylneopine was then separated from the other alkaloids by tlc systems a, c, and d.

Table V. R_f Values

Compound	System a	b	c	d
Thebaine	0.65		0.65	0.40
Codeine	0.40	0.25	0.15	0.10
Codeinone	0.55	0.40	0.35	
6-Methylcodeine	0.70	0.50	0.40	0.35
6-Methylneopine	0.70		0.30	0.25
Neopinone	9.53	0.40		
Morphine	0.30	0.10		
6-Phenylcodeine	0.80			
Neopine	0.40	0.25		

Chromatography Systems. Column Chromatography. Woelm silica gel for tlc (no binder), deactivated by standing 24 hr in shallow pans exposed to air, was used. Eluting solvents were $CHCl_3$ (80), CH_3OH (20), NH_4OH (0.5%).

Thin Layer Chromatography. With Camag silica gel for tlc, the solvent systems were (a) $CHCl_3$ (80), CH_3OH (20), NH_4OH (0.05%); (b) $CHCl_3$ (25), dioxane (60), ethyl acetate (10), NH_4OH (5).[31] With Merck aluminum oxide G for tlc, the solvent system were (c) benzene (70), $CHCl_3$ (15), acetone (15), shaken with 3.5% NH_4OH;[32] (d) cyclohexane (8), $CHCl_3$ (2), acetone (6). R_f values are given in Table V.

Gas chromatography was performed on 6 ft × 6 mm glass columns, packed with 4.5% OV1 on Aeropak 30 (100–120 mesh), at 210° using argon at 60 ml/min as the carrier gas and a hydrogen flame detector. The retention times were for 6-methylcodeine, 4 min 15 sec; thebaine, 6 min 45 sec; codeine, 4 min 45 sec; 6-phenylcodeine, 21 min; morphine, 5 min 30 sec.

(31) J. A. Steele, *J. Chromatogr.*, **19**, 300 (1965).

(32) L. Vignoli, J. Guillot, F. Gouezo, and J. Catalin, *Ann. Pharm. Fr.*, **24**, 461 (1966).

Mass Spectrometry in Structural and Stereochemical Problems. CCXI.[1] The Effect of Structural Variations on the Electron Impact Induced Fragmentations of Steroid Hydrocarbons[2]

George Eadon,[3] S. Popov,[4] and Carl Djerassi*

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305. *Received May 27, 1971*

Abstract: The electron impact induced fragmentations of *D*-homoandrostane (IV) and *D*-homopregnane (III) are qualitatively similar to those of androstane (II) and pregnane (I), respectively. In particular, the characteristic ring D and ring A cleavages were subjected to close scrutiny, and were demonstrated to be mechanistically completely analogous to the processes observed in the parent compounds. In contrast, the mass spectral behavior of *D*-norandrostane (VI) and *D*-norpregnane (V) differs markedly from that of the parent compounds. For example, the very abundant *m/e* 218 ion is produced without reciprocal hydrogen transfer in these compounds. The site of charge localization, and thus of ring cleavage, is strikingly dependent on the structure of the steroid framework, particularly in the androstane series. This dependence is rationalized on the basis of relief of steric strain.

The electron impact induced fragmentation of steroids possessing an alkyl substituent at C-17 (*e.g.*, pregnane, I) characteristically involves extensive fragmentation about ring D, a process indicated schematically by the wavy line in structural formula I. The diagnostic importance of this fragmentation pattern was recognized over 15 years ago; the process reveals the molecular weight of the substituent at C-17.[5] Recently,[6] extensive deuterium labeling experiments have

(1) For paper CCX, see A. N. Yeo and C. Djerassi, *J. Amer. Chem. Soc.*, **94**, 482 (1972).

(2) Financial support by the National Institutes of Health (Grants AM 12758 and AM 04257) is gratefully acknowledged.

(3) National Institutes of Health Predoctoral Fellow, 1968–1971.

(4) Recipient of an IREX fellowship while on leave (1970–1971) from the Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia.

(5) P. de Mayo and R. I. Reed, *Chem. Ind. (London)*, 1481 (1956).

(6) L. Tökés, G. Jones, and C. Djerassi, *J. Amer. Chem. Soc.*, **90**, 5465 (1968).